Winter 2010

Phenotypic characterization of palmitoyltransferase mutants of Arabidopsis thaliana

Ulkucan Kaplan

University of New Hampshire, Durham

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PHENOTYPIC CHARACTERIZATION OF PALMITOYLTRANSFERASE MUTANTS OF ARABIDOPSIS THALIANA

BY

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Bachelor of Science, Gazi University, 2005

THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Genetics

December, 2010
This thesis has been examined and approved.

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ACKNOWLEDGMENTS

I would like to thank to my advisor Dr. Estelle Hrabak, whose guidance and support to develop an understanding of the subject. I am also thankful to my graduate committee members Dr. Thomas Davis and Dr. Kevin Culligan for all edits and advices. I also would like to thank Dr. Christopher Neefus, Dr. Subhash Minocha, Dr. Charles Walker and Dr. Swathi Anuradha Turlapati for their technical support. I wish to thank to my lab-mates Megan Thomson and Jack Lepine, for their help and friendship. It is a pleasure to thank those who made this thesis possible to complete.

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ABSTRACT

PHENOTYPIC CHARACTERIZATION OF PALMITOYLTRANSFERASE MUTANTS OF ARABIDOPSIS THALIANA

by

Ulkucan Kaplan

University of New Hampshire, December, 2010

This study seeks to understand the effects of loss of palmitoylation catalyzed by PAT14 on the phenotype of Arabidopsis thaliana. In this project, I focused on two T-DNA insertion mutants of the PAT14 gene in Arabidopsis thaliana: pat14-1 and pat14-2. PAT14 is one of 23 PAT genes encoding palmitoyltransferase (PAT) proteins in Arabidopsis thaliana. PAT proteins are responsible for palmitoylation, one of several cellular mechanisms to modify proteins by lipidation. The purpose of this research was to describe the effect of T-DNA insertion mutations in the PAT14 gene on the phenotype of Arabidopsis thaliana plants. Characterization of palmitoyltransferase mutants of Arabidopsis thaliana indicated that lack of palmitoylation by PAT14 has a role in plant growth, affecting leaves and roots, seed production and germination.
1. INTRODUCTION

Lipid modifications of proteins include the addition of fatty acids, isoprenoids and cholesterol (Nadolski and Linder, 2007). Some of these modifications occur in the cytoplasm or on the cytoplasmic side of membrane while others occur in the lumen of secretory pathway organelles. N-myristoylation, prenylation and S-palmitoylation are three common lipid modifications of proteins, which occur in the cytoplasm or on the cytoplasmic side of membrane (Resh, 1999). N-myristoylation is the covalent addition of the 14-carbon fatty acid myristate to an N-terminal glycine residue by an amide linkage (Resh, 1999). Prenylation is the addition of 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid groups to a C-terminal cysteine residue via a thioether linkage (Zhang and Casey, 1996). S-palmitoylation, which is referred as palmitoylation hereafter, involves the addition of a 16-carbon saturated fatty acyl chain, palmitate, to cysteine residues via a thioester linkage (Charollais and Van Der Goot, 2009).

**Palmitoylation**

Palmitoylation is unique because it is the only reversible lipid modification. The reversibility of palmitoylation allows it to regulate the activity of proteins differently than myristoylation and prenylation (Charollais and Van Der Goot, 2009).
Palmitoylation of proteins occurs by two different mechanisms (Smotrys and Linder, 2004). In a few cases, palmitoylation occurs spontaneously and palmitate is transferred from acyl-CoA to the target cysteine residue nonenzymatically (Berthiaume et al., 1994; Corvi et al., 2001) but in the majority of cases, palmitoylation is mediated by an enzyme (Linder and Deschenes, 2003). The enzymes responsible for the addition and removal of palmitate are protein acyl transferases (PAT) and protein palmitoyl thioesterases (PPT) (Varner et al., 2003). Palmitoylation occurs on both peripherally-associated and integral membrane proteins (Smotrys and Linder, 2004).

**Palmitoyltransferases**

Palmitoyltransferases were first discovered in *Saccharomyces cerevisiae* (yeast) by identification of Erf2 that is responsible for palmitoylation of Ras2 and of Akr1 that palmitoylates the casein kinase Yck2 (Lobo et al., 2002; Roth et al., 2002). Like all S-acyl transferases, Erf2 and Akr1 are integral membrane proteins and share a conserved cysteine-rich domain (CRD) with an embedded DHHC (aspartate-histidine-histidine-cysteine) motif (Mitchell et al., 2006; Smotrys and Linder, 2004): \(\text{C}_2\text{C}_9\text{H}\_\text{C}_2\text{C}_4\text{DHHC}_5\text{C}_4\text{N}_3\text{F}.\)

The importance of the DHHC domain is provided by studies that suggest that this domain is required for the transfer of palmitate (Mitchell et al., 2006). Since mutations in the DHHC domain abolish PAT activity, this domain is essential for mediating the palmitoylation reaction (Fukata et al., 2006).
Palmitoyltransferases are found in all eukaryotes and multiple genes encoding PAT proteins have been identified in many organisms. For example, the *Saccharomyces cerevisiae* genome encodes seven DHHC-CRD-containing proteins (Winzeler et al., 1999) while humans have 23 genes (Mitchell et al., 2006; Ohno et al., 2006; Tsutsumi et al., 2008) and Drosophila has 22 (Bannan et al., 2008). *Arabidopsis thaliana*, which was used in this project, has 23 genes encoding PATs (Hemsley et al., 2005). The presence of multiple PAT genes in an organism indicates that these enzymes likely have numerous functions in cells (Mitchell et al., 2006).

All palmitoyltransferases are integral membrane proteins with multiple transmembrane domains (TMDs). The previously-mentioned yeast Erf2 protein has four TMDs while Akr1 has six (Nadolski and Linder, 2007). In addition, Akr1 has a series of amino-terminal ankyrin repeats which often have a role in protein-protein interactions although it is unknown whether the ankyrin domain has any function when Akr1 palmitoylates Yck2 (Smotrys and Linder, 2004). Figure 1 illustrates the predicted orientation of Erf2 and Akr1 proteins within the membrane.
Figure 1. Predicted membrane orientation of the Erf2 and Akr1 palmitoyltransferases (Nadolski and Linder, 2007). Thick black lines = transmembrane domains; thin black lines = hydrophilic amino acid chains; thin grey lines = DHHC-CRD domains.
**Functions of Palmitoyltransferases**

PAT proteins have important and diverse roles in eukaryotic cells and interfering with palmitoylation of proteins often leads to phenotypic changes in the mutant organisms. The following illustrate some of the functions of palmitoylation.

A role for palmitoylation in protein trafficking between organelles and the plasma membrane has been demonstrated. The trafficking of H-Ras and N-Ras from either the ER or Golgi to the plasma membrane requires palmitoylation; these proteins accumulate in the early secretory pathway when palmitoylation is blocked (Chiu et al., 2002; Rocks et al., 2005; Roy et al., 2005).

Palmitoyltransferases have a role in targeting proteins to lipid rafts, which are membrane microdomains enriched in cholesterol and sphingolipids. Many proteins involved in signaling pathways are targeted to these highly-ordered membrane regions by acylation and it is hypothesized that these proteins form functional complexes in the lipid rafts. For example, palmitoylation is critical for the membrane binding of G-protein α subunits and their association with the β and γ subunits to form functional complexes (Dunphy, et al., 2001). In addition, palmitoylation of LAT and CD8β transmembrane proteins also require palmitoylation for localization to lipid rafts (Arcaro et al., 2000; Zhang, et al., 1998).

Palmitoylation can regulate protein turnover. Regulation of the half-life of yeast chitin synthase Chs3 is an example of the role of palmitoylation as a quality
control checkpoint. Palmitoylation of Chs3 by the Pfa4 palmitoyltransferase acts as an indicator for proper folding. When palmitoylation is prevented, Chs3 accumulates in the ER instead of trafficking to plasma membrane and so, chitin deposition is decreased on the cell surface. This study suggested that palmitoylation has a role in the ER quality control of membrane proteins and is one way to modulate protein stability (Lam et al., 2006). Palmitoylation also affects the degradation of Lcb4 (sphingoid long-chain base kinase). Lcb4 is a soluble protein palmitoylated by Akr1 in yeast. The down regulation of Lcb4 during stationary phase does not occur in akr1 mutants, so palmitoylation negatively regulates the half life of this protein (Kihara et al., 2005).

Increasing the membrane affinity of a soluble protein is a common effect of palmitoylation and indicates that palmitoylation can affect protein localization (Nadolski and Linder, 2007). Akr1 is a palmitoyltransferase required for cell mating, normal cell morphology and regulation of G-protein-coupled-receptor (GPCR) trafficking (Dohlman and Thorner, 2001). Akr1 palmitoylates the casein kinase Yck2 in yeast. In wildtype yeast, the soluble Yck2 protein is anchored to the plasma membrane by its palmitoyl group. In the absence of Akr1, Yck2 is mislocalized to the cytosol (Feng and Davis, 2000). Similarly, yeast sphingoid long-chain base kinase Lcb4 is palmitoylated by Akr1 PAT protein. When wildtype and mutant yeast cells, which were stained anti-Lcb4 antibodies, were examined by immunofluorescence microscopy, akr1 mutant cells showed a widespread intracellular fluorescence pattern indicative of cytosolic localization of Lcb4 while Lcb4 was observed on the cell perimeter in wildtype cells. Therefore,
reduced palmitoylation in mutants decreased the membrane association of Lcb4 protein (Kihara et al., 2005).

The analysis of palmitoyltransferase mutations in humans also indicated the impact of palmitoylation in several diseases. Huntingtin-interacting protein (HIP14, also known as DHHC17) is a PAT and palmitoylates huntingtin protein (Huang et al., 2004). This study demonstrated a protective role of palmitoylation in preventing protein aggregation. The accumulation of intracellular protein aggregates due to misfolding of huntingtin with long polyQ-repeats causes Huntington's disease. Mutant huntingtin (is a poor substrate for HIP14) and thus is more prone to aggregation (Landles and Bates, 2004). The PATs DHHC15 and DHHC9 are implicated in X-linked mental retardation (Mansour et al., 2005; Raymond et al., 2007) while the PAT DHHC8 has been associated with schizophrenia (Mukai et al., 2004). Also, several studies demonstrated that some human PAT proteins have roles in various cancers (Ducker et al., 2004; Mansilla et al., 2007; Oyama et al., 2000; Yamamoto et al., 2007).

The previous examples illustrate the wide variety of roles for palmitoylation in yeast and humans; however, the study of palmitoylation in plants is still relatively limited. TIP1 (Tip Growth Defective1) from Arabidopsis is the only plant PAT that has been characterized (Hemsley et al., 2005). The TIP1 gene encodes a palmitoyltransferase with similarities to yeast Akr1 and human HIP14 proteins because of their amino-terminal ankyrin repeat. Three TIP1 mutants, tip1-1, tip1-2 and tip1-3, had smaller plant sizes and shorter and wider root hairs than wildtype plants. Conversely, over-expression of the TIP1 gene caused longer...
root hairs in Arabidopsis plants (Hemsley et al., 2005). This study also suggested that TIP1 is involved in pollen tube growth.

**Importance of This Study**

Although we now have knowledge of a wide variety of roles for palmitoylation in yeast and human cells, characterization of plant PATs is still relatively limited. Therefore, identification of plant PAT mutants and their subsequent characterization will contribute to the study of protein palmitoylation in plants.

**Arabidopsis thaliana and pat Mutants**

*Arabidopsis thaliana*, a small flowering plant and widely-used model organism, was chosen as the subject of this project. Arabidopsis has a relatively small genome (approximately 135 Mb) and is the first plant whose genome was sequenced (The Arabidopsis Information Resource, 2010; The Arabidopsis Genome Initiative, 2000). Arabidopsis has a rapid life cycle, produces many seeds, has a compact size and is easy to grow in the laboratory, making it an excellent tool for molecular biology and genetic studies.

The size of the Arabidopsis *PAT14* gene (At3g60800) is 2427 basepairs (bp) not including the promoter and has 7 exons (Figure 2). Two mutants created by insertion of transferred DNA (T-DNA) into the *PAT14* gene were used in this study. Both insertion sites are in the first exon. The *pat14-1* T-DNA insertion occurred 49 bp after the translation start codon (Figure 3) while the *pat14-2* T-DNA insertion is 44 bp after the translation start codon (Figure 4). Thus, the
distance between T-DNA insertion sites is only 5 bp. The mutant lines were obtained from the SALK (Alonso et al., 2003) and GABI-KAT (Rosso et al., 2003) mutant collections. Plants containing homozygous pat14-1 and pat14-2 alleles were previously identified (A. Argyros and E. Hrabak, unpublished data). Both mutant alleles are in the Columbia ecotype of *Arabidopsis thaliana* which was used as the control for all experiments.
Figure 2. Genomic structure of the *PAT14* gene.
Figure 3. *PAT14* gene with *pat14-1* T-DNA insertion. T-DNA inserted 49 bp after the translation start codon. (J. Lepine, unpublished data)
Figure 4. PAT14 gene with pat14-2 T-DNA insertion. T-DNA inserted 44 bp after the translation start codon.

(J. Lepine, unpublished data)
The purpose of this research is to describe the effect of T-DNA insertion mutations in the *PAT14* gene on the phenotype of *Arabidopsis thaliana* plants. In this project, I focused on two T-DNA insertion mutants of the *PAT14* gene in *Arabidopsis thaliana*: *pat14-1* and *pat14-2*. *PAT14* is one of 22 *PAT* genes encoding palmitoyltransferase (PAT) proteins in *Arabidopsis thaliana*. PAT proteins are responsible for palmitoylation, which is one of several cellular mechanisms to modify proteins by lipidation (Smotrys and Linder, 2004).

I hypothesize that *pat14-1* and *pat14-2* mutants will have similar phenotypes since their T-DNA insertion sites are close to each other in the same exon of the *PAT14* gene. Two types of comparisons were done: comparing phenotypes of wildtype and mutant plants and comparing phenotypes of mutants to each other. Wildtype and mutant plants were examined for phenotypic differences using a variety of assays. These assays characterized leaf and root phenotypes, seed production, and germination. Specifically, the assays examined leaf lesions, leaf length, leaf number, leaf area, chlorophyll and anthocyanin content, leaf phenolic compounds, root length, number of lateral roots, number of siliques, number of seeds per silique, seed yield, seed size, and germination time.
2. MATERIALS AND METHODS

Plant Growth

Depending on the assay, Arabidopsis thaliana was grown in soil or aseptically on basal medium. For growth in soil, seeds were sown on the surface of a mixture of 1 part of Metro Mix 360 (SunGro Horticulture, Bellevue, WA) and 1 part of graded horticultural perlite (Whittemore Inc., Lawrence, MA) in 6 cm square pots. Pots were placed in 27 cm x 53.5 cm flats in a randomized pattern and covered with a plastic dome until germination occurred. Seedlings were thinned to 1 seedling per pot in the second week after planting. Two growth chambers, growth chamber 1 and 2, were used to grow plants in soil. Growth conditions of both chambers were 21°C with an 18-hour photoperiod. However, growth chamber 2 has smaller size, accommodating only 10 flats on 2 shelves and also has less distance between shelves than growth chamber 1, which can hold 72 flats on 6 shelves.

For growth on defined medium under aseptic conditions, seeds were sterilized in 70% ethanol with one drop of 10% Triton X-100 with occasional shaking followed by one wash in 100% ethanol containing one drop of Triton X-100 and one wash in 100% ethanol. All washes were for 5 minutes. The seeds were air dried in a laminar flow hood and sown on petri plates containing 30 ml of basal medium (1 X MS). Basal medium was composed of 0.44% Murashige-
Skoog (MS) Medium Plus Vitamins (Caisson Labs, Catalog Number: MSP0506), 0.05% MES (2-(N-morpholino) ethanesulfonic acid; Sigma Chemical Co., Catalog Number: M2933) and 0.8% plant tissue culture grade Phytoblend (Caisson Labs, Catalog Number: PTC001), pH 5.7–5.8. After stratification for 3 days, plates were placed vertically in growth chamber 3 at 25°C with a 12 hour photoperiod.

*Arabidopsis thaliana* plants were grown in three different growth chambers during this research. The dates of each assay and name of the growth room are in Table1.
Table 1. Dates and growth chamber for each assay. Growth chamber
1: Conviron, TCR144, 21°C, 18-hour photoperiod, Rudman penthouse; Growth
Chamber 2: Pervical, AR-60L, 25°C, 12-hour photoperiod, Rudman penthouse; Growth
Chamber 3: Harris, 25°C, 12-hour photoperiod, 126A Rudman.

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Leaf Measurements

Leaf measurements were performed on 3, 4, 5, 6 and 7 week-old-plants grown in soil. Twelve plants of each genotype were measured at each time point. Plants in pots were photographed with a Pentax K100D digital camera before the intact shoots were removed. The intact, detached shoots were photographed. Finally, cotyledons and leaves were removed, arrayed in order from oldest to youngest, and photographed. A ruler was included in the photographs to assist with calibration of subsequent measurements.

Leaf length and leaf area were measured using Image J software (National Institutes of Health). Cotyledons were included in these measurements. Leaf length was measured along the mid-vein of the leaf from the top of the petiole to the tip of each individual leaf or cotyledon using the Segmented Line tool. The leaf lengths for each plant were averaged and these values were averaged for each genotype at each time point. For leaf area, the area of each leaf, not including the petiole, was measured using the Threshold tool. The sum of the leaf areas for each plant was calculated and averaged for each genotype at each time point. The number of leaves per plant was counted including the cotyledons and then averaged for each genotype at each time point. For leaf lesions and flowering time, leaves, not including cotyledons, were observed every day and notes describing the appearance of the plants were recorded.
**Root Measurements**

Root length and number of lateral roots were measured in plants grown on vertically-oriented plates of basal medium. Root length was measured on 3, 5, 7, 9 and 11 day-old plants while lateral roots were counted on days 7, 9 and 11. Each plate included 5 seeds of two different genotypes. Three genotypes (wildtype, *pat14-1* and *pat14-2*) were tested in all combinations, with each genotype placed on the left side of half of the plates and on the right side of the other half of the plates. Five replicates were used for each arrangement for a total of 30 plates.

Root length was measured from the end of the hypocotyl (defined as the root-shoot junction) to the end of the root tip using the Segmented Line tool in Image J and averaged for each genotype in each plate at each time point. Both pre-emergent and emerged lateral roots were counted by scanning along the length of the root using a dissecting microscope and the values were averaged for each genotype at each time point.

**Germination Time**

Thirty seeds of three genotypes (wildtype, *pat14-1* and *pat14-2*) were placed on one plate and 12 plates were used for a total of 360 seeds per genotype. The order in which the genotypes were placed on each plate was randomized. The seeds were observed every 12 hours for 2 days using the dissecting microscope. The number of germinated seeds, where germination is
defined as the first appearance of the radicle tip from the testa (seed coat), was recorded for each genotype for each plate.

**Seed Production**

The number of siliques per plant and the number of seeds in a silique were examined on in the same experiment. Plants were grown separately from those used for leaf measurements described previously. Twelve pots of plants were grown for each genotype (wildtype, pat14-1 and pat14-2). Siliques were counted two weeks after flowering (defined as the first open flower on the plants) and also at 7 weeks after germination. Seeds per silique were counted 3 weeks after flowering. Five siliques per plant were selected for seed counts and averaged per genotype.

Total seed yield was determined for 12 plants per genotype. A seed collector was used to collect the seeds. The seed collectors were handmade using transparency film, tape and staples. The collectors were fitted on the pots in the seventh week before any siliques had matured. The final seed harvest was done after 14 weeks. Seeds from each plant were weighed individually and averaged per genotype.

Seed size measurements were done using 100 harvested seeds of wildtype, pat14-1 and pat14-2 genotypes. Seeds were chosen randomly and photographed under a dissecting microscope. The length and width of each seed were measured using the Straight Line tool in the Image J program and averaged
separately per genotype. Both developed and undeveloped seeds were included in these measurements.

**Pigment Analysis**

Pigment assays were performed on 2, 3 and 4 week-old-plants using the three oldest rosette leaves of each plant. Plants were grown separately from those used for leaf measurements or seed production. Five replicates were used per genotype at each time point. Before pigment analysis, pictures of intact shoots and of leaves ordered from oldest to youngest were taken as described above. The three oldest leaves, without petioles, of each plant were weighed. To estimate total chlorophyll and anthocyanin content, a protocol modified from Gitelson et al., 2009 was used. Briefly, the leaves were ground with a mortar and pestle using 200 µl methanol per mg of plant tissue. To prevent chlorophyll pheophytization, grinding was done in the presence of a pinch of CaCO₃ (Gitelson et al., 2009). Homogenates were centrifuged for 5 minutes at 10000 rpm. For chlorophyll, the supernatants were transferred to new tubes and extracts were assayed spectrophotometrically at 665 and 652 nm. Total chlorophyll was calculated as described by Lichtenthaler (1987). For the determination of anthocyanin concentration, the extracts were adjusted to 1 % HCl and absorbance was read at 530 nm. An anthocyanin absorption coefficient of 30 mM⁻¹ · cm⁻¹ was used (Strack and Wray, 1989).
**Microscopy**

Phenolic compound accumulation was based on observation of autofluorescent compounds using a fluorescence microscope. Observations were performed on 14, 18 and 22 day-old plants. Five plants of each genotype were used at each time point. Rosette leaves were cleared by modification of the method of Shipton and Brown, 1962. The shoots were immersed in alcoholic lactophenol (1 volume of phenol: glycerol: lactic acid: water (1:1:1:1) and 2 volumes of ethanol). Five ml of alcoholic lactophenol was used for approximately 1 cm² of plant tissue which was vacuum evacuated for 15 minutes. Then leaves were placed at 65°C and incubated for 30 min to remove chlorophyll. Finally, the leaves were transferred to fresh alcoholic lactophenol for an additional 2-24 h at room temperature. The cleared leaves were mounted in 25% glycerol and examined under epifluorescent illumination (peak excitation at 488 nm and peak emission at 518 nm; Adam and Somerville, 1996).

**Measurements and Statistical Analyses**

All measurements were done with Image-J software (National Institutes of Health). Systat (Systat Software Inc.) was used for statistical analysis. A Tukey’s range test was used in conjunction with an ANOVA to detect significant differences. Means were compared in all combinations. P value for significance was 0.05 in all comparisons.
3. RESULTS

The characterization of the phenotypes of plants with mutations in the \textit{PAT14} palmitoyltransferase gene was performed to understand the role of palmitoylation in plant growth. Characterization assessed leaf and root organs, seed production, germination, pigment concentration and phenolic compound accumulation. For each assay, the phenotypes of wildtype and mutant plants which were grown in the same environment were compared. In each experiment, multiple plants or seeds were used for each genotype and most experiments were replicated at least two times.

**Characterization of Leaf Phenotypes**

Characterization of leaf phenotypes of \textit{pat14} mutants was done to determine the effects of mutations in the \textit{PAT14} gene on leaf appearance and size. Preliminary observations indicated that \textit{pat14-1} and \textit{pat14-2} mutant plants looked different than wildtype plants, with smaller plant size and more lesions on the rosette leaves. Therefore, characterization of leaf phenotypes included detailed observation of lesions on rosette leaves and measurement of total leaf number, leaf length and leaf area during 7 weeks of growth. I expected that \textit{pat14} mutants would have different leaf phenotypes than wildtype, but similar phenotype to each other.
Total leaf number and measurements of leaf length and leaf area were performed at five time points: weeks 3, 4, 5, 6 and 7. Five observations of the plants throughout their life cycle enabled me to look at the effect of the mutations over time. Measurements did not begin until week 3 and ended at week 7 because preliminary experiments indicated that the plants did not exhibit any visible phenotypic differences before the third week and many leaves had senesced by the eighth week.

Leaf assays were done two times and the data from both assays are shown. Growth chamber 1 was used for Assay 1 while growth chamber 2 was used for Assay 2. At each time point, plants were photographed while they were still in the pots (data not shown). After the shoots were detached from the plants, all shoots for each genotype were photographed as a group. An overview of leaf assays is shown in Figure 5.

From Figures 6-10, it appears that there are phenotypic differences between wildtype and pat mutants. Wildtype plants appear to be larger and healthier than mutant plants starting at about week 4. The leaf assays were chosen to enable quantitation of these differences. To facilitate subsequent measurements, the leaves were removed and arranged in order from oldest to youngest. The leaf alignment of each plant was used to quantitate leaf number, leaf length and leaf area. The alignments of leaves from representative plants are shown in Figures 11-25.
Figure 5. Overview of leaf assays. Each plant was photographed in its pot and, after the shoots were detached, a group picture of all 12 plants of each genotype was taken at each time point. Leaves were detached from each shoot and arranged in order from oldest to youngest. The leaf order image was used to determine leaf number, length and area.
Figure 6. Shoots of 3-week-old plants. A) Assay 1; B) Assay 2
Figure 8. Shoots of 5-week-old plants. A) Assay 1; B) Assay 2
Figure 9. Shoots of 6-week-old plants. A) Assay 1; B) Assay 2

pat14-2

pat14-1

wildtype

A)

B)
Figure 10. Shoots of 7-week-old plants. A) Assay 1; B) Assay 2
Figure 11. Leaves from representative 3-week old wildtype plants. Leaves are arranged from oldest (left) to youngest (right). A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 12. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 4-week-old wildtype plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 13. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 5-week-old wildtype plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 14. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 6-week-old wildtype plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 15. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 7-week-old wildtype plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2
Figure 16. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 3-week-old *pat14-1* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 17. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 4-week-old *pat14-1* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 18. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 5-week-old *pat14-1* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 19. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 6-week-old pat14-1 plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 20. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 7-week-old *pat14-1* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 21. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 3-week-old *pat14-2* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
**Figure 22.** Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 4-week-old *pat14-2* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 23. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 5-week-old *pat14-2* plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 24. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 6-week-old pat14-2 plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Figure 25. Leaves from representative leaves are arranged from oldest (left) to youngest (right) from 7-week-old pat14-2 plants. A) Plant 1 from Assay 1; B) Plant 2 from Assay 1; C) Plant 1 from Assay 2; D) Plant 2 from Assay 2.
Number of Leaves per Plant

The number of rosette leaves, including cotyledons, per plant was counted and averaged per genotype at each time point. I expected that the leaf number of wildtype plants and *pat14* mutant plants would be different.

For Assay 1, there was no significant difference in leaf number between the three genotypes at weeks 3 and 4 (Figure 26A). At week 5, wildtype plants had more leaves than mutants and there was no significant difference between mutants. Wildtype and *pat14-2* plants had more leaves than *pat14-1* plants at weeks 6 and 7 and there was no significant difference between wildtype and the *pat14-2* mutant (Figure 26A). When I compared the leaf number for each genotype over the course of the experiment, all three genotypes showed similar trends (Figure 26B and Table 2). The number of leaves of wildtype and *pat14-2* plants increased between 3 and 4 weeks, but did not change for the following three weeks. The *pat14-1* followed a similar pattern until week 6, but the leaf number decreased significantly between weeks 6 and 7.

For Assay 2, there was no significant difference in leaf number between the three genotypes at weeks 3 and 4. Wildtype and *pat14-2* plants had more leaves than *pat14-1* plants at week 5. However, at weeks 6 and 7, there was no difference between mutants although they had lower leaf number than wildtype plants (Figure 27A). A comparison of leaf number for each genotype during the 5 week experiment revealed that there was no significant difference in the week-to-
week comparisons for three genotypes. The leaf numbers of wildtype and pat14-1 plants decreased between weeks 6 and 7 while there was no difference for pat14-2 (Figure 27B and Table 3).

The results of Assay 2 were unexpected based on my knowledge of Arabidopsis growth. When the pictures of plant groups in Figures 8, 9 and 10 were compared for Assay 1 and Assay 2, the plants from Assay 2 appeared visibly smaller than Assay 1 plants at weeks 5, 6 and 7. This obvious difference in plant size was reflected in leaf number data. The average maximum number of leaves per wildtype plant in Assay 1 was 18.00 while the average maximum number of leaves per wildtype plant in Assay 2 was 13.25. In both experiments, wildtype plants had greatest leaf numbers from 4 to 6 weeks. Regardless of absolute differences in leaf number, the trend for leaf accumulation and decline was similar in Assays 1 and 2.

The general trend in both Assays 1 and 2 was that pat14 mutants had fewer leaves than wildtype at weeks 5, 6 and 7. There are several reasons that the pat mutant plants might have fewer leaves at later time points. First, their leaves may senesce earlier than wildtype. Second, the mutants may grow slower than wildtype. Third, the mutants may flower earlier than wildtype. Plants do not form any additional rosette leaves after transitioning from vegetative to the reproductive phase (Adams et al., 2003).
To assess the second and third possibilities, the day on which the first flower opened was recorded. Data is presented as the percent of plants flowering each day because the number of plants observed changed from week to week since some of the plants were used for leaf measurement assays each week. Because of the experimental design, the data could not be analyzed statistically.

For Assay 1, one wildtype plant and two pat14-2 plants were the first to flower at day 30. All plants of these two genotypes flowered by day 40, although flowering of pat14-2 appeared to occur slightly earlier than wildtype. The first pat14-1 plant flowered on day 34 and all pat14-1 plants were flowering by day 43 (Figure 28A). For Assay 2, one wildtype plant was the first to flower at day 25 and the first pat14-2 plant flowered on day 26 followed by three pat14-1 plants on day 27. All wildtype plants completed flowering by day 36 while pat mutants completed flowering by day 37 (Figure 28B). The flowering curve of the three genotypes appeared to be similar in Assay 2.

Based on leaf number and flowering time data, there was no correlation between flowering time and leaf number of genotypes in Assay 1 and Assay 2. Thus, differences in flowering time do not explain the significant difference in leaf number between wildtype and pat14 mutants. Therefore, the reason for fewer leaves in pat14 mutants must be earlier senescence or slower growth.
Figure 26. Number of leaves of wildtype, *pat14-1* and *pat14-2* plants in Assay 1. (A) Comparison of the three genotypes at weekly intervals. The three genotypes were compared at each week using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p≤0.05. (B) Average leaf number by genotype from weeks 3-7. See Table 2 for statistical analysis.
Table 2. Statistical analysis summary of the data in Figure 26B. Time points were compared for each genotype using Tukey’s range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at p≤0.05. Grey fill indicates the week-to-week comparisons.

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Figure 27. Number of leaves of wildtype, *pat14-1* and *pat14-2* plants in Assay 2. (A) Comparison of the three genotypes at weekly intervals. The three genotypes were compared at each week using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p<0.05$. (B) Average leaf number by genotype from weeks 3-7. See Table 3 for statistical analysis.
Table 3. Statistical analysis summary of the data in Figure 27B. Time points were compared for each genotype using Tukey’s range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at p≤0.05. Grey fill indicates the week-to-week comparisons.

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Figure 28. Percentage of plants flowering from days 29-45. A) Assay 1; B) Assay 2.
Leaf Length

Average leaf length was determined weekly for each genotype. Leaf length was measured from the leaf tip to the petiole along the midvein and did not include the petiole. Leaf length was measured individually for each leaf. The length of all leaves of each plant was totaled and averaged and then these values were averaged for each genotype at each time point. Since average leaf length is affected by the number of leaves, I expected to measure leaf length would help comparison of plant sizes of three genotypes.

In Assay 1, there was no difference in the average leaf length of the three genotypes at weeks 3 and 4; however, the average leaf length of the three genotypes was different at weeks 5, 6 and 7 (Figure 29A). Wildtype plants had greater average leaf length than either of the mutants at weeks 5, 6 and 7 while the pat14-2 mutant had longer leaf length than the pat14-1 mutant. The average length of the wildtype leaves significantly increased from week to week for the duration of the experiment, reflecting the increasing size of the plants seen previously in Figures 6-10 (Figure 29B and Table 4). For pat14-1, the average weekly leaf length stopped increasing on week 5 while the average leaf length of pat14-2 plants increased until week 6 and did not change between 6 and 7 weeks (Figure 29B and Table 4).

In some respects, Assay 2 was very similar to Assay 1 as there was no difference between the average leaf length of the three genotypes at weeks 3
and 4 and wildtype plants had longer leaf length than *pat* mutants at weeks 5, 6 and 7 (Figure 30A). However, there was no significant difference between the mutants at any time point (Figure 30A). Also, the average leaf length of wildtype increased only between weeks 4 and 5 indicating that plants were not growing as robustly after week 5 as in Assay 1. For mutants, average leaf length did not change significantly from week-to-week but did increase when longer time spans are compared (Figure 30B and Table 5). Overall, the plants grew similarly until week 5, and then the leaf length of *pat14* mutant plants was smaller compared to wildtype plants in both assays.

Although the two assays showed similar trends when comparing whether wildtype and mutants were significantly different in average leaf length, there appeared to be some growth differences in the two assays. The largest average leaf length for wildtype leaves in Assay 1 was 3.5 cm at week 7 while in Assay 2, the largest value for wildtype average leaf length was only about 2 cm and it did not occur at week 7 but was maintained for weeks 5, 6 and 7. This data indicated that plants in Assay 2 did not grow as well as Assay 1 plants. The plants grew similarly until week 5 in both experiments, and then plants in Assay 2 seemed to stop growing for unknown reasons (Figure 29-30).

As with the number of leaves per plant, there was no statistical difference in average leaf length between wildtype and *pat* mutant plants at 3 and 4 weeks of age. In general, wildtype plants had higher leaf length than mutants at weeks
5, 6 and 7 in both assays. Average leaf length data correlate with leaf number data since wildtype plants had more leaf number and longer leaf length than *pat14* mutants at most weeks.
Figure 29. Average leaf length of wildtype, pat14-1 and pat14-2 plants in Assay 1. (A) Comparison of three genotypes at weekly intervals. The three genotypes were compared at each week using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p<0.05. (B) Average leaf length by genotype from weeks 3-7. See Table 4 for statistical analysis.
Table 4. Statistical analysis summary of the data in Figure 29B. Time points were compared for each genotype using Tukey's range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at \( p \leq 0.05 \). Grey fill indicates the week-to-week comparisons.

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Figure 30. Average leaf length of wildtype, \textit{pat14-1} and \textit{pat14-2} plants in Assay 2. (A) Comparison of three genotypes at weekly intervals. The three genotypes were compared at each week using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at \(p<0.05\). (B) Average leaf length by genotype from weeks 3-7. See Table 5 for statistical analysis.
**Table 5.** Statistical analysis summary of the data in Figure 30B. Time points were compared for each genotype using Tukey’s range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at \( p \leq 0.05 \). Grey fill indicates the week-to-week

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**Leaf Area**

Total leaf area was calculated by measuring and summing the area of all cotyledons and rosette leaves. The total leaf area of each plant was averaged per genotype at each timepoint. I expected that *pat14* mutant plants would have smaller leaf area than wildtype plants based on visual observations.

In Assay 1 at weeks 3 and 4, there was no significant difference between the leaf area of any of the genotypes (Figure 31A). At weeks 5 through 7, the area of wildtype plants was significantly larger than the mutants. There was no significant difference between leaf area of *pat* mutants at weeks 5 and 7 while *pat14-2* plants were larger than *pat14-1* plants at week 6 (Figure 31A). The area of wildtype plants increased significantly from week 4 to 5 and from week 5 to 6 while the leaf area of *pat14* mutants did not change significantly from week-to-week over the 7 weeks of the experiment (Figure 31B and Table 6).

The overall trends for Assay 2 are very similar to Assay 1. There was no significant difference between genotypes at weeks 3 and 4 (Figure 32A). At weeks 5, 6 and 7, wildtype plants were larger than either mutant, although there was no difference between the *pat* mutants (Figure 32A). The area of wildtype plants increased from weeks 3 to 5 and 4 to 5 while the plant area of *pat* mutants did not change during the 7 week experiment (Figure 32B and Table 7).

Although the trends for leaf area are very similar in the two assays, wildtype plants in Assay 1 reached a maximum area of approximately 6000 mm$^2$. 
while in Assay 2 the largest area was only about 2500 mm$^2$. This is consistent with both the smaller leaf number and smaller average leaf length noted previously for Assay 2.
Figure 31. Leaf areas of wildtype, pat14-1 and pat14-2 plants in Assay 1. 
A) (A) Comparison of three genotypes at weekly intervals. The three 
genotypes were compared at each week using Tukey’s range test in 
conjunction with an ANOVA. Different letters indicate that the averages 
are statistically significant at $p \leq 0.05$. (B) Average leaf length by genotype 
from weeks 3-7. See Table 6 for statistical analysis.
Table 6. Statistical analysis summary of the data in Figure 31B. Time points were compared for each genotype using Tukey’s range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at \( p \leq 0.05 \). Grey fill indicates the week-to-week comparisons.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Wildtype</th>
<th>pat14-1</th>
<th>pat14-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 vs. 4</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3 vs. 5</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3 vs. 6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3 vs. 7</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>4 vs. 5</td>
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<td>4 vs. 6</td>
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<td>4 vs. 7</td>
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<td>5 vs. 6</td>
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<td>5 vs. 7</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>6 vs. 7</td>
<td>-</td>
<td>-</td>
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</table>
Figure 32. Leaf areas of wildtype, pat14-1 and pat14-2 plants in Assay 2. (A) Comparison of three genotypes at weekly intervals. The three genotypes were compared at each week using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p<0.05$. (B) Average leaf length by genotype from weeks 3-7. See Table 7 for statistical analysis.
**Table 7.** Statistical analysis summary of the data in Figure 32B. Time points were compared for each genotype using Tukey’s range test in conjunction with an ANOVA. + indicates significant difference while - indicates no significant difference at $p<0.05$. Grey fill indicates the week-to-week comparisons.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Wildtype</th>
<th>pat14-1</th>
<th>pat14-2</th>
</tr>
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<tbody>
<tr>
<td>3 vs. 4</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>3 vs. 5</td>
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<td>3 vs. 6</td>
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<td>6 vs. 7</td>
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</table>
**Leaf Lesions**

Plants grown in pots were observed every day and changes in leaf appearance were scored in three categories: pale yellow chlorotic lesions, dry brown necrotic lesions, or purple anthocyanic lesions. Plants were scored as positive for chlorosis, necrosis or anthocyanin if they had any of a particular type of lesion on one or more leaves and the percentage of plants with each type of lesion on any leaf was calculated per day. Cotyledons were not included in lesion observations. An example of the three categories of leaf lesion is shown in Figure 33.

Since the number of plants observed changed from week to week because some of the plants were used for leaf measurement assays each week, data is presented as the percent of plants flowering each day. The data could not be analyzed statistically because of the experimental design.
Figure 33. Example of the three categories of lesions scored on Arabidopsis rosette leaves.
Appearance of Chlorotic Lesions - Chlorosis was observed on leaves as pale yellow or yellow-white areas, usually in spots or in interveinal regions. In nature, chlorosis is caused by insufficient production of chlorophyll or by growth conditions. Plant stress factors like temperature, radiation, pollutants may cause degradation of chlorophyll (Halliwell and Gutteridge, 1985; Hendry et al., 1987; Schoch et al., 1984; Sisson and Caldwell, 1976). In Arabidopsis and many other plants, chlorosis also occurs normally as the oldest leaves begin to senesce.

In Assay 1, chlorosis first appeared on some wildtype leaves on day 21 but appeared on the first mutant leaves on day 19. All wildtype plants had at least one chlorotic area by day 33 while pat mutants had at least one chlorotic lesion by day 24 (Figure 34). In Assay 2, chlorotic lesions appeared earlier for all three genotypes compared to Assay 1, again indicating that plants in Assay 2 were not growing as well as in Assay 1. In Assay 2, chlorotic lesions were first observed on mutants on day 12 and observed on wildtype on day 15. All wildtype plants had at least one chlorotic lesion by day 30 while chlorosis was observed on all pat14-1 mutants on day 24 and on pat14-2 plants on day 23 (Figure 35). Chlorosis was also appeared sooner on mutant plants (Figure 11-25), and 100% of mutants had chlorotic lesions 7-9 days earlier than wildtype plants in both assays.
Figure 34. Percentage of plants displaying leaf chlorosis in Assay 1. Plants were scored as chlorotic if they had chlorotic lesion on one or more leaves.
Figure 35. Percentage of plants displaying leaf chlorosis in Assay 2. Plants were scored as chlorotic if they had a chlorotic lesion on one or more leaves.
Appearance of Anthocyanic Lesions - Purple lesions were observed on the leaves of all genotypes but mutants developed purple lesions earlier than wildtype. It is likely that the purple color of the lesions is due to anthocyanin accumulation. Anthocyanin is a plant flavonoid synthesized by the shikimic acid pathway. Different environmental stresses like drought, radiation and osmotic stress can induce anthocyanin production (Chalker-Scott, 1999). Age and nutrient limitation are also correlated with anthocyanin accumulation (Diaz et al., 2005).

In Assay 1, wildtype plants had the first purple lesions on day 27, but mutants had similar lesions earlier, on day 23 (Figure 36). In Assay 2, these lesions were first observed on the leaves of wildtype on day 21; however, they were first observed on the pat14-1 plants on day 17 and on the pat14-2 plants on day 18 (Figure 37). For both assays, there appeared to be more purple lesions on the leaves of mutant plants and they appeared earlier than on wildtype plants (Figures 11-25). By 7 weeks, %100 of mutants had anthocyanic lesions in both Assays 1 and 2 while wildtype plants did not since some wildtype plants never developed anthocyanic lesions.
Figure 36. Percentage of plants with anthocyanic leaf lesions in Assay 1. Plants were scored as anthocyanin positive if they had at least one purple lesion on one or more leaves.
Figure 37. Percentage of plants with anthocyanic leaf lesions in Assay 2. Plants were scored as anthocyanin positive if they had at least one purple lesion on one or more leaves.
Appearance of Necrotic Lesions - Necrosis is a result of the death of plant cells and occurs normally in older leaves of wildtype plants.

In Assay 1, necrosis first appear on some wildtype leaves on day 31 while they were first observed on the pat14-1 plants on day 28 and on the pat14-2 plants on day 30 (Figure 38). In Assay 2, necrotic lesions were first observed on the wildtype leaves on day 26, but appeared on the first mutant leaves on day 22 (Figure 39). Necrotic lesions were observed earlier on pat mutants than on wildtype plants in both assays (Figures 11-25). However, in Assay 2, necrotic lesions appeared earlier on all three genotypes compared to Assay 1 (Figures 38 and 39). In both Assays 1 and 2, % 100 of plants of pat mutants had necrotic lesions by 7 weeks, while some wildtype plants never developed necrotic lesions.

In summary, chlorotic, anthocyanic and necrotic lesions appeared on all pat mutants while all wildtype plants did not have anthocyanic and necrotic lesions by 7 weeks. Plants were grown under the same light and temperature conditions in this experiment, yet mutants developed chlorotic, anthocyanic and necrotic leaf lesions sooner than wildtype. These results indicated that mutations in the PAT14 gene contributed to lesion formation perhaps as a result of metabolic imbalances that led to insufficient chlorophyll production, cell death and/or accumulation of anthocyanin.
Figure 38. Percentage of plants with necrotic leaf lesions in Assay 1. Plants were scored positive for necrosis if they had a necrotic lesion on one or more leaves.
Figure 3.9. Percentage of plants with necrotic leaf lesions in Assay 2. Plants were scored positive for necrosis if they had a necrotic lesion on one or more leaves.
Chlorophyll and Anthocyanin Quantitation

Chlorophyll is the major pigment required for photosynthesis in plants. In this assay, total chlorophyll (chl a + b) were measured. Figures 6-10 showed that wildtype plants usually had darker green leaves compared to the pat14 mutants perhaps indicating higher levels of chlorophyll. Anthocyanin is a class of flavonoids that are synthesized via the shikimate pathway (Chalker-Scott, 1999). The accumulation of this pigment in Arabidopsis leaves is normally indicative of stress.

Chlorotic and anthocyanic lesions were observed in leaves of all three genotypes but appeared earlier on the pat14-1 and pat14-2 mutants compared to wildtype. It was expected that the amount of chlorophyll and anthocyanin pigments would be different between wildtype and pat mutants. I expected the concentration of chlorophyll to decrease in plants having chlorotic lesions while anthocyanin concentration should increase in plants with multiple anthocyanic lesions. To confirm these expectations, anthocyanin and chlorophyll pigments were extracted and quantitated.

For the measurements, the three oldest rosette leaves of wildtype, pat14-1 and pat14-2 plants were chosen because lesions first appear on the older leaves of plants. Since lesion appearance usually started around 3 weeks after planting (Figures 34-39), 2-, 3- and 4-week-old plants were used. The plants had similar phenotypes to previous leaf assay plants although leaf number, length and area were not recorded. However, chlorotic lesions appeared earlier than anthocyanic
lesions, at around weeks 2, 3 and 4. Mutant plants developed lesions sooner than wildtype plants.

Estimation of chlorophyll and anthocyanin pigments was done two times. Plants were grown in growth chamber 2 in both Assay 1 and Assay 2. These assays were not done at the same time as the previous leaf measurements, meaning that Assay 1 for chlorophyll was not done on the same plants used for leaf measurements in Assay 1, described previously.

In Assay 1, the chlorophyll concentration of *pat14-1* plants was lower than wildtype and *pat14-2* at week 2 while there was no difference between wildtype and *pat14-2*. At weeks 3 and 4, chlorophyll concentration was not significantly different between the three genotypes (Figure 40A). The amount chlorophyll in wildtype plants did not change over the course of three weeks. For mutants, the concentration increased between weeks 2 and 3, but did not change from week 3 o 4 (Figure 40B).

In Assay 2, the concentration of chlorophyll in the three genotypes was not different at weeks 2 and 3. However, the chlorophyll concentration of wildtype plants was higher than *pat* mutants at week 4 (Figure 41A), while there was no difference between *pat14-1* and *pat14-2*. The chlorophyll concentration of wildtype plants did not change significantly from weeks 2 to 4, but it significantly decreased between weeks 2 and 3 for *pat14-1* mutants and between weeks 3 and 4 for *pat14-2* plants (Figure 41B).
Figure 40. Chlorophyll concentration of wildtype, *pat14-1* and *pat14-2* plants in Assay 1. A) Comparison of the three genotypes at weekly intervals. B) Chlorophyll concentration by genotype for weeks 2, 3 and 4. Comparisons were done using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p<0.05.
Figure 41. Chlorophyll concentration of wildtype, pat14-1 and pat14-2 plants in Assay 2. A) Comparison of the three genotypes at weekly intervals. B) Chlorophyll concentration by genotype for weeks 2, 3 and 4. Comparisons were done using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p≤0.05.
For Assay 1, anthocyanin concentration of the three genotypes was not different at weeks 3 and 4, and only differed significantly between wildtype and *pat14-1* plants at week 2 (Figure 42A). Anthocyanin concentration of wildtype plants did not change over the three weeks of the experiment while anthocyanin increased for both *pat14* mutants between weeks 2 and 3, which was expected from visual observations (Figure 42B).

For Assay 2, anthocyanin concentration was not significantly different between genotypes at weeks 2, 3 or 4 (Figure 43A). In the comparison of weeks, anthocyanin concentration of wildtype and *pat14-2* plants was not different between weeks 2 and 3, and also between weeks 3 and 4 while it decreased from week 2 to 4. For *pat14-1* plants, the concentration decreased from week 2 to 3, but did not change between weeks 3 and 4 (Figure 43B).
Figure 42. Anthocyanin concentration of wildtype, pat14-1 and pat14-2 plants in Assay 1. A) Comparison of the three genotypes at weekly intervals. B) Anthocyanin concentration by genotype for weeks 2, 3 and 4. Comparisons were done using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p≤0.05.
Figure 43. Anthocyanin concentration of wildtype, pat14-1 and pat14-2 plants in Assay 2. A) Comparison of the three genotypes at weekly intervals. B) Anthocyanin concentration by genotype for weeks 2, 3 and 4. Comparisons were done using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$.
The plants used in both Assays 1 and 2 did not have any chlorotic, anthocyanic or necrotic lesions 2 weeks after planting. By week 3, chlorotic lesions first appeared on pat14 mutant leaves while they were not apparent on wildtype leaves. Although plants from both assays had similar symptoms around the same time points, data from Assay 1 and 2 was significantly different for chlorophyll and anthocyanin concentrations. The reason for these discrepancies is unknown. Maybe the conditions in the growth room caused stress and affected the production of chlorophyll and anthocyanin. The expectation was chlorophyll concentration would be higher in wildtype plants than pat14 mutants especially at weeks 3 and 4 because chlorotic lesions usually first appeared between weeks 2 and 3 after planting in mutants (Figures 34 and 35). On the other hand, since anthocyanic leaves first appeared around 3 week in mutants (Figures 36 and 37), I expected that pat14 mutants would have high anthocyanin concentration than wildtype at 3 and 4 weeks. The results from both Assays did not match expectations.
**Production of Phenolic Compounds in Leaves**

Phenolic compounds are one of the most important secondary metabolite groups in plants. Phenolic compounds are characterized by one or more aromatic benzene rings with one or more hydroxyl groups and are synthesized primarily from the cinnamic acids (Dixon and Paivan, 1995). Phenolic compounds have important roles in plant defense and the production of these compounds is often induced by external stimuli, such as cold, nutrient limitation, infection or injury (Ruiz et al., 2003; Sakihama and Yamasaki, 2002; Takahama and Oniki, 2000). Phenolic compounds can accumulate in different tissues and cells such as the epidermal and subepidermal cells of leaves, the central vacuole of guard cells, shoots, cell walls, or external surfaces of plant organs (Cuadra and Harbone, 1996; Moskowitz and Hrazdina, 1981; Ozimina, 1979; Schnabl et al., 1986, 1989; Schnitzler et al., 1996; Strack et al., 1988; Weissenbock et al., 1986). Anthocyanins are of the flavonoids (Chalker-Scott, 1999) and flavonoids are phenolic compounds. Therefore, the detection of phenolic compounds may be used to observe the early development of anthocyanic lesions. Some of the anthocyanins are fluorescent (Markakis, 1985), so if fluorescent compounds accumulate in the cells of the *pat* mutants it may indicate that the mutations cause plant stress or induce plant defense responses.

Leaves were observed by fluorescence microscopy on 14, 18 and 22 day-old-plants, which were grown in growth chamber 1, and five replicates were used for each genotype in each time point. Fluorescence microscopy was used since several groups of phenolic compounds are intensely fluorescent when excited by
UV or visible radiation of the correct wavelength (Ibrahim and Barron, 1989; Monici, 2005; Veit et al., 1993). Plant cells contain many kinds of autofluorescent compounds, most of which contain aromatic rings. Day 14 after planting was determined as the first observation time because plants do not yet have any visual symptoms.

Fluorescent compound accumulation was not observed on the wildtype, pat14-1 and pat14-2 leaves at day 14 (data not shown). Fluorescent compounds were detected on the leaves of some 18-day-old plants of each of the three genotypes, but the number of plants having these compounds was not correlated with phenotype since regions of fluorescence were observed on many plants: two of five wildtype plants, one of five pat14-1 plants and four of five pat14-2 plants (Figure 44). At day 22, the number plants with accumulation of fluorescent compounds increased for pat mutants while this number did not change for wildtype plants. Fluorescent compound accumulation was observed for four of five pat14-1 plants and all pat14-2 plants (Figure 45). Thus pat14 mutations do not appear to cause unusual amounts of phenolic compounds to accumulate in leaves, at least not during the first 3 weeks of growth.
Figure 45. Detection of autofluorescent compounds on leaves of 22-day-old plants
Seed Production

Arabidopsis produces many seeds per plant. The seeds develop in siliques, which are the fruits of Arabidopsis thaliana. The characterization of seed production was done because mutant plants were smaller than wildtype, so I wondered that if that affected seed production. Seed production was quantitated by counting the number of siliques and the number of seeds per siliquae and by measuring total seed yield and seed size. Growth chamber 1 was used to grow plants for all seed production assays. Silique numbers, seeds per siliquae and seed yields were repeated two times and the composite data is shown for each assay since the results of the replicated assays were not significantly different. Data of each independent assay is shown in the Appendix.

Number of Siliques

The number of siliques on plants of all three genotypes was counted at two different developmental stages on the same plants: two weeks after flowering and at 7 weeks of age. Siliques were counted on twelve plants of each genotype. Two weeks after flowering, statistical analysis showed that wildtype plants had more siliques than pat14-1 and pat14-2 while the number of siliques was not different between mutants (Figure 46).

Counting the number of siliques per plant at week 7 indicated that the average siliquae number of wildtype and pat14-2 plants was greater than pat14-1. Furthermore, there was no significant difference between wildtype and pat14-2 (Figure 47) and this result was unexpected. I expected that wildtype plants would
have more siliques than *pat14* mutants because the plant size of mutants was smaller than wildtypes. However, 7-week old wildtype and *pat14-2* plants had a similar number of siliques. The reason may be that *pat14-2* plants continued flowering and producing siliques longer than wildtype and *pat14-1* plants although this type of data was not recorded.
Figure 46. Average number of siliques per plant 2 weeks after flowering. Data is from two independent experiments. The three genotypes were compared using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at \( p \leq 0.05 \).
**Figure 47.** Average number of siliques per genotype on 7-week-old plants. Data is from two independent experiments. The three genotypes were compared using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p<0.05.
**Number of Seeds per Silique**

The number of seeds in mature siliques, which were yellow and contained mature brown seeds, was counted 3 weeks after flowering. Before this time, there were almost no mature siliques on the plants, especially the mutants.

Five siliques were picked randomly and the seeds in each silique were counted and averaged per plant. It was expected that wildtype plants would have more seeds per silique than *pat14* mutants and the results supported this expectation. Wildtype plants had significantly more seeds per silique than mutants, and also *pat14-2* plants had more seeds per silique than *pat14-1* (Figure 48).
Figure 48. Average number of seeds per silique 3 weeks after flowering. Data is from two independent experiments. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. 
**Seed Yield**

Based on the reduced number of siliques and fewer seeds per silique in the *pat14* mutants, I anticipated that the mutants would have less total seed production. Twelve plants per genotype were grown. After harvesting seeds using seed collectors, seeds from each plant were weighed individually and the weights were averaged for each genotype. The amount of seeds produced by wildtype, *pat14-1* and *pat14-2* plants was significantly different. Wildtype plants produced more seeds than the mutants and also the amount of seeds from *pat14-2* plants was more than from *pat14-1* (Figure 49). These results indicate that loss of PAT14 palmitoylation affected seed production of *Arabidopsis thaliana* plants.
Figure 49. Yield of seeds from wildtype, *pat14-1* and *pat14-2* plants. Data is from two independent experiments. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. 

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</tr>
<tr>
<td><em>pat14-2</em></td>
<td>160</td>
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</table>
**Seed Size**

Wildtype plants produced more seed weight than mutants, and also had more total siliques and more seeds per silique. I next asked if the sizes of mutant seeds were smaller than wildtype seeds. For the seed size measurements, 100 seeds were used per genotype and all seeds (developed and undeveloped seeds) were considered. Since *Arabidopsis* seeds are oval (Figure 50), the length and width of seeds were measured separately and then averaged for each genotype. The data showed that the length of wildtype and *pat14-2* seeds was not different, but these genotypes had significantly longer seeds than *pat14-1* (Figure 51). Wildtype seeds were significantly wider than seeds of either *pat14* mutants (Figure 52).
**Figure 50.** Mature seeds of wildtype *Arabidopsis thaliana* and the *pat14* mutants.
Figure 51. Average length of wildtype, \textit{pat}14-1 and \textit{pat}14-2 mature seeds. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. 
Figure 52. Average width of wildtype, *pat14-1* and *pat14-2* mature seeds. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at \( p \leq 0.05 \).
Germination

Germination is the process in which a plant emerges from a seed and begins growth. By assaying germination time, I will determine whether a palmitoyltransferase mutation affects this early developmental stage. Germination time of seeds and total number of germinated seeds per genotype were compared. The germination assay was performed two times and the averaged data from both experiments is presented. The data from each independent assay is given in Appendix.

I counted the number of germinated seeds per genotype at each time point. A total of 720 seeds were used per genotype in the two assays. Growth chamber 3 was used and the experimental design for this assay is shown in Figure 53. The germinated seeds were counted every 12 hours for 2 days and the percent germination was calculated for each genotype. In the first 12 hours, the number of germinated seeds was low and was not significantly different between genotypes (Figure 54). Most of the seeds had germinated at the 24-hour time point, the number of germinated seeds of wildtype plants was significantly higher than both mutants while *pat14-1* had more germinated seeds than *pat14-2* at this time point. The number of germinated seeds of the *pat14-2* mutant was also different than the other genotypes after 36-hours, but there was no difference between wildtype and the *pat14-1* mutant. There was no significant difference in the total number of germinated seeds for each genotype at the end of 48 hours (Figure 55).
Figure 53. Experimental design for seed germination assay. Thirty seeds (grey dots) of each genotype were placed on petri plates (black rectangles). Twelve plates were used with the order of genotypes randomized on each plate.
Figure 54. Number of germinated seeds per genotype. Data is from two independent experiments. The three genotypes were compared at each time point using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at \( p \leq 0.05 \).
Figure 55. Total number of germinated seeds after 2 days. Data is from two independent experiments. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p<0.05$. 
Root Length

The data represented so far on the characterization of the pat14 mutants indicates that PAT14 palmitoylation is important for leaf phenotype, seed production and germination. The root is the organ which takes water and nutrients from the soil for plant growth. If the mutants are affected in root growth, that might be the reason for all of the phenotypes described previously. The length of primary roots was measured on plants grown on basal medium on vertically-oriented plates for 11 days in growth chamber 3. Figure 56 shows the experimental design for the root assay. Pictures of the plates were taken on days 3, 5, 7, 9 and 11 (Figure 57) and root lengths were measured. The experiment was stopped at day 11 because the roots reached the bottom of the plates after this day and further measurements could not be made.

Root length was measured from the end of the hypocotyl (root-shoot junction) to the root tip. The transition from hypocotyl to root was recognized by the lack of chlorophyll in the root and by the presence of root hairs forming at the root shoot junction at later time points.
Figure 56. Experimental design for root assays. Five seeds (grey dots) of each genotype were placed on petri plates (black rectangles); two genotypes were used per plate in all possible combinations. For each combination, five replicate plates were used.
Figure 57. Representative wildtype and pat14 mutants grown on petri dishes. Plants were photographed 3, 5, 7, 9 and 11 days after stratification.
Root length assays were performed three times. Since root length data from the three assays were significantly different, data from each independent assay is shown in Figures 58 and 59.

In Assay 1, there was no significant difference in root length between the genotypes at day 3. The pat14-1 mutant was significantly shorter than wildtype at days 5, 7, 9 and 11 while there was no significant difference between wildtype and pat14-2 (Figure 58A). The root length of the three genotypes was not different at days 3, 5 and 7 in Assay 2. On days 9 and 11, there was no difference between wildtype and pat14 mutants while pat14-1 had shorter root length than pat14-2 (Figure 58B). In Assay 3, the root length of three genotypes was the same at each time point (Figure 58C). Although there were differences between the results of the three assays, Assay 2 and Assay 3 were more similar to each other than to Assay 1.

Plants of all genotypes showed a significant increase in root length from one time point to the next in Assays 1, 2 and 3. This indicated that roots of all genotypes continue to elongate during the first 11 days of growth (Figure 59).
Figure 58. Root length of wildtype, *pat14-1* and *pat14-2* plants in Assays 1, 2 and 3. The three genotypes were compared at each time point using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at \( p \leq 0.05 \). A) Assay 1; B) Assay 2; C) Assay 3.
Figure 59. Comparasion of average root lengths by genotype in Assays 1, 2 and 3. Time points were compared for each genotype using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. A) Assay 1; B) Assay 2; C) Assay 3.
Lateral Roots

The lateral root initials, pre-emergence lateral roots and emerged lateral roots were counted using a dissecting microscope on days 7, 9 and 11. Earlier time points were not evaluated because few lateral root initials were present before day 7. Since primary root lengths did differ between genotypes, two possible outcomes might be predicted for lateral roots: First, if root phenotypes are not the cause of the leaf phenotypes then lateral roots would not differ between genotypes, similar to the result with the primary roots. If root phenotypes are the cause of the leaf phenotypes, and primary roots did not show a defect, then a phenotype would be expected in the lateral roots.

Lateral roots were counted on the same plants that were used for root length measurement in two of the three root length experiments. In the first root experiment, only root length was measured and lateral roots were not counted. The data from both lateral root assays was not significantly different. Therefore, the averaged data from assays is presented. Data from each independent experiment is in the Appendix. The number of lateral roots in each genotype was not significantly different on day 7. However, wildtype plants had more lateral roots than both pat14 mutants on days 9 and 11 (Figure 60). Also, the number of lateral roots significantly increased for each genotype from one timepoint to the next (Figure 61). Results indicated that even though pat14 mutants formed fewer lateral roots on days 9 and 11 than the wildtype seedlings, the mutants did continue to develop lateral roots over the course of the experiment. This result
supported the scenario that the *pat14* mutation affects lateral root formation early in development but does not affect early primary root growth.
Figure 60. Lateral root number of wildtype, pat14-1 and pat14-2 plants. Data is from two independent experiments. The three genotypes were compared at each time point using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p<0.05$. 
**Figure 61.** Comparison of lateral roots by genotype. Data is from two independent experiments. Time points were compared for each genotype using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. 
A summary of each independent assay is presented in Tables 9-13. Significant differences are indicated using different letters. Based on the data from leaf, seed and germination assays, wildtype plants were generally larger and more productive than pat14 mutants. However, root length measurement data did not support this statement. Although pat14-1 and pat14-2 seedlings had fewer lateral roots than wildtype, root lengths did not differ between genotypes at most time points. Since root lengths were compared at only early developmental time points, and leaf and seed phenotypes were measured at later times, any effects of the mutations may not be apparent in roots at these early times.
Table 9. Summary for the statistical analyses of leaf assays. Data from Assay 1 and Assay 2 are shown separately. Different letters indicate that the averages are statistically significant at p≤0.05. Statistics were performed independently for each time point and experiment.

<table>
<thead>
<tr>
<th></th>
<th>Assay 1</th>
<th></th>
<th>Assay 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wildtype</td>
<td>pat14-1</td>
<td>pat14-2</td>
<td>wildtype</td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>3 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>5 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>B</td>
<td>AB</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>7 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf number</td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>leaf length</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>leaf area</td>
<td>A</td>
<td>B</td>
<td>B</td>
<td>A</td>
</tr>
</tbody>
</table>
Table 10. Summary of statistical analyses of pigment assays. Data from Assay 1 and Assay 2 are shown separately. Different letters indicate that the averages are statistically significant at $p<0.05$. Statistics were performed independently for each time point and experiment.

<table>
<thead>
<tr>
<th></th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wildtype</td>
<td>pat14-1</td>
</tr>
<tr>
<td>2 weeks</td>
<td>chlorophyll</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>anthocyanin</td>
<td>A</td>
</tr>
<tr>
<td>3 weeks</td>
<td>chlorophyll</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>anthocyanin</td>
<td>A</td>
</tr>
<tr>
<td>4 weeks</td>
<td>chlorophyll</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>anthocyanin</td>
<td>A</td>
</tr>
</tbody>
</table>
Table 11. Summary of statistical analyses of siliques and seed measurements.
Data from Assay 1 and Assay 2 are shown separately. Different letters indicate that the averages are statistically significant at $p<0.05$. Statistics were performed independently for each time point and experiment.

<table>
<thead>
<tr>
<th></th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>wildtype</strong></td>
<td><strong>pat14-1</strong></td>
</tr>
<tr>
<td>siliques number</td>
<td>A B B</td>
<td>A B B</td>
</tr>
<tr>
<td>(2-wk after flowering)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siliques number</td>
<td>A B A</td>
<td>A A A</td>
</tr>
<tr>
<td>(7-week)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seeds in siliques</td>
<td>A B C</td>
<td>A B B</td>
</tr>
<tr>
<td>seed yield</td>
<td>A B C</td>
<td>A B B</td>
</tr>
<tr>
<td>seed length</td>
<td>A B A</td>
<td>- - -</td>
</tr>
<tr>
<td>seed width</td>
<td>A B B</td>
<td>- - -</td>
</tr>
</tbody>
</table>
Table 12. Summary of statistical analyses of germination assays. Data from Assay 1 and Assay 2 are shown separately. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. Statistics were performed independently for each time point and experiment.

<table>
<thead>
<tr>
<th>Time</th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wildtype</td>
<td>pat14-1</td>
</tr>
<tr>
<td>12 hours</td>
<td>germination</td>
<td>A</td>
</tr>
<tr>
<td>24 hours</td>
<td>germination</td>
<td>A</td>
</tr>
<tr>
<td>36 hours</td>
<td>germination</td>
<td>A</td>
</tr>
<tr>
<td>48 hours</td>
<td>germination</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td><strong>total germinated seeds</strong></td>
<td>A</td>
</tr>
</tbody>
</table>
Table 13. Summary of root measurements. Data from Assay 1, Assay 2 and Assay 3 are shown separately. Different letters indicate that the averages are statistically significant at \( p \leq 0.05 \). Statistics were performed independently for each time point and experiment.

<table>
<thead>
<tr>
<th></th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wildtype</td>
<td><em>pat14-1</em></td>
<td><em>pat14-2</em></td>
</tr>
<tr>
<td>3 days</td>
<td>root length</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>5 days</td>
<td>root length</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>7 days</td>
<td>root length</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>7 days</td>
<td>lateral root</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 days</td>
<td>root length</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>9 days</td>
<td>lateral root</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11 days</td>
<td>root length</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>11 days</td>
<td>lateral root</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
4. DISCUSSION

The phenotypic identification of palmitoyltransferase mutants provided the opportunity to determine the roles of palmitoylation in plant life cycle. A potential role of palmitoylation in Arabidopsis thaliana growth was identified by characterization of phenotypes of two mutants of the PAT14 gene. T-DNA insertion mutations in the PAT14 gene caused smaller plant size with more leaf lesions compared to wildtype Arabidopsis. The data supported my hypothesis that the pat mutants have different phenotypes than wildtype plants. I also hypothesized that pat14-1 and pat14-2 plants have similar phenotypes. Most of the assays supported this hypothesis although sometimes pat14-1 phenotypes were stronger than pat14-2.

Previous information about palmitoyltransferase function in plants is relatively limited. Although there are many studies on PAT genes and proteins in other organisms, TIP1 is the only plant PAT protein which has been identified in the literature (Hemsley et al., 2005). This study included both the molecular identification of the Arabidopsis TIP1 gene and phenotypic characterization of tip1 mutants. Three mutant alleles (tip1-1, tip1-2 and tip1-3) were found to have similar phenotypes when compared to wildtype Arabidopsis thaliana. Mutant plants were smaller than wildtype plants and had shorter and wider root hairs.
Also, mutations in the TIP1 gene affected pollen tube growth in Arabidopsis. In this project, pat14 mutants had smaller plant size, similar to tip1 mutants, but the lesions that appeared on pat14 mutant leaves were not reported for tip1 mutant plants. Since root hair growth and pollen tube formation were not examined in this research, the effect of the pat14 mutation on these processes is unknown. From studies on these two palmitoyltransferases, we know that defects in palmitoylation can have effects on Arabidopsis phenotypes and that mutations in different PATs affect different processes since pat14 and tip1 mutants have different phenotypes.

The completion of the Arabidopsis genome sequence (The Arabidopsis Genome Initiative, 2000) provided the opportunity to study the function of individual genes. In addition, large collections of Arabidopsis insertional T-DNA mutants are available to assess in understanding gene function by the study of mutant phenotypes. In this research, mutant lines were acquired from publicly-available mutant collections (Alonso et al., 2003; Rosso et al., 2003). Both collections used insertional mutagenesis by T-DNA from Agrobacterium tumefaciens to create loss of function mutations. T-DNA insertion mutations are important for studies of gene function in Arabidopsis and have several advantages. First, T-DNA insertions are chemically and physically stable and heritable from one generation to the next. Second, T-DNA can be used as a marker for identification of the mutation (Radhamony et al., 2005). This research concentrated on the PAT14 gene because plants containing homozygous pat14-1 and pat14-2 alleles were previously found to display a phenotype that looked
markedly different from wildtype plants (A. Argyros and E. Hrabak, unpublished data). The T-DNA insertions in *pat14-1* and *pat14-2* occurred in the first exon. The phenotype of *pat14-1* mutants has been complemented previously using the wildtype *PAT14* gene (E. Hrabak, unpublished data). Using two *pat14* mutants also provided the opportunity to compare the phenotypes with each other. One conclusion from this research was that *pat14-1* plants sometimes showed more severe phenotypes than *pat14-2* plants. This was an unexpected result since the T-DNA insertion mutations were in the same exon and the distance between the insertion sites was only 5 base pairs. The most likely explanation is that the T-DNAs used for mutagenesis may affect the phenotype of the mutant plants. The T-DNA vectors used to create the *pat14-1* and *pat14-2* mutants have different structures but are functionally very similar (Figure 62). The GABI-KAT T-DNA inserted in the *pat14-2* mutant has a 1'2' dual promoter, which regulates transcription initiation bi-directionally. The 1' promoter initiates transcription of *sul* gene in *pat14-2*; *sul* is plant selectable marker gene which should not affect phenotype of plant. The 2' side of the promoter faces out from the *PAT14* gene toward the tRNA^{Lys} gene which is about 1 kb away. The impact of the 2' promoter on transcription of this tRNA gene is unknown.

The 35S constitutive promoters, which face the left border in the T-DNA of *pat14-1* and the right border in the T-DNA of *pat14-2*) are capable of causing high levels of transcription in plants. Both promoters direct transcription toward the downstream portion of the *PAT14* gene. It has been demonstrated that 35S promoter in the Salk T-DNA is capable of driving expression of the downstream
sequences (Ulker et al., 2008). Based on these statements, 35S (in pat14-2) and 35S (in pat14-1) promoters can affect the PAT14 protein expression, but where translation starts and how affects the PAT14 protein is unknown. Since 2' promoter (in pat14-2) is pointing away from the PAT14 gene, it is unlikely to affect the PAT14 protein.

The SALK T-DNA in pat14-1 has a kanamycin-resistance cassette, consisting of a Nos promoter, nptII gene, and NosT terminator, which terminates the expression of the adjacent gene. nptII is a selectable marker for plant transformation like sul gene and is unlikely to have an effect on the phenotype. bla is also a selectable marker gene, but is only active in bacteria, so it cannot affect the plant phenotype (Figure 62).

The slight variation in phenotype between the two pat14 mutants may be due to differences in the T-DNAs or may be due to other factors which are unknown at this time. Overall though, the phenotypes of pat14-1 and pat14-2 are very similar, indicating that these mutations provide a consistent picture of the effect of loss of PAT14 palmitoylation. More detailed knowledge of the precise structure of the T-DNA inserts may be necessary if we want to understand the subtle differences in phenotype between pat14-1 and pat14-2.
Figure 62. Structure of T-DNA in *pat14-1* and *pat14-2* mutants. Images are from Ulker et al. (2008). T-DNAs are oriented in the same way that they inserted into *PAT14* gene. A) T-DNA inserted in *pat14-1* mutant and obtained from the SALK collection (Alonso et al., 2003) B) T-DNA inserted in *pat14-2* mutant and obtained from the GABI-Kat collection (Rosso et al., 2003).
For phenotypic characterization, a variety of measurements and assays were chosen based on the appearance of mutant plants. One obvious feature of the palmitoyltransferase mutants was smaller rosette leaves with many yellow and purple patches. Mutant plants also appeared smaller than wildtype overall. I found that wildtype plants had more leaves, longer average leaf length and a greater plant area than pat mutants especially from 4 weeks onward. The cause of the smaller leaf size can be the smaller cells or fewer cells in the pat14 mutants. There were more chlorotic, anthocyanic and necrotic lesions on the mutants relative to wildtype plants and they appeared earlier. Leaf chlorosis and necrosis is normal in plants and is associated with senescence but these symptoms can also be a result of stress.

Palmitoylation has several documented effects in eukaryotes. Chitin synthase is palmitoylated by Pfa4 in yeast, and lack of palmitoylation triggers decreased chitin deposition on the cell surface (Lam et al., 2006). Palmitoylation also affects the trafficking of Ras proteins between ER or Golgi and plasma membrane and Ras proteins accumulate in the early secretory pathway when their palmitoyltransferase is inactivated (Chiu et al., 2002; Rocks et al., 2005; Roy et al., 2005). The relationship between palmitoylation and some human diseases has been demonstrated (Ducker et al., 2004; Huang et al., 2004; Mansilla et al., 2007; Mansouri et al., 2005; Mukai et al., 2004; Raymond et al., 2007; Oyama et al., 2000; Yamamoto et al., 2007).

Some of the substrates for PATs identified in yeast or humans might also be PAT targets in plants. For example, yeast Akr1 palmitoylates casein kinase 2
(Yck2) (Feng and Davis, 2000) and sphingoid long-chain base kinase (Lcb4).

Both the casein kinase 1 family and sphingoid long-chain base (LCB) kinase are also found in plants (Imai and Nishiura, 2005; Stone and Walker; 1995). Although we do not know the substrates for PAT14, it is clear that lack of palmitoylation can lead to phenotypes in many organisms.

Quantitation of chlorophyll and anthocyanin were done to make a connection between leaf appearance and pigment concentration. Although the experiments were repeated two times, the data from the assays were not reproducible so I could not conclude that lack of PAT14 palmitoylation affected the pigment concentration in plants. Usually symptoms were observed starting at about 3-weeks of age, but the assays did not reflect what I could see by eye in the pat14 mutant plants. Based on visual observations, symptoms were apparent in older plants (i.e., paler green mutant plants, more purple lesions on mutants). Problems with growth conditions might be the reason for these unexpected results because a different growth chamber (growth chamber 2) was used in the pigment assays that may have had higher humidity or different light quality than growth chamber 1. As a result, if I was going to repeat this experiment, I would use the same growth chamber for all experiments.

The induction of phenolic compound biosynthesis can be based on stress factors such as low temperature, infection, injury, low nutrients and metal toxicity (Diaz, et al, 2001; Ruiz et al., 2003; Sakihama and Yamasaki, 2002; Takahama and Oniki, 2000). Production of phenolic compounds was investigated to determine whether lack of palmitoylation triggers a plant defense response.
Other mutants that form spontaneous lesions in the absence of pathogens have been described. The Arabidopsis LSD1 gene, which encodes a novel zinc finger protein, is required for negative regulation of programmed cell death (Dietrich et al., 1997; Kliebenstein, 1999; Mateo et al., 2004). The mutants of this gene developed dead cells on the leaves under cold stress (Huang, 2010). A mutation in the Arabidopsis HLM1 gene, which encodes a cyclic nucleotide-gated channel (CNGC4), caused smaller and thicker leaves and shorter petioles than wildtype plants and also had spontaneous lesion development. Lesions were not visible by eye before week 3 of growth, but could be seen with a microscope. Under a fluorescence microscope, autofluorescent phenolic compound accumulation was observed around the lesions (Balague et al., 2003). Accumulation of phenolic compounds is a hallmark of the plant defense response. However, my data indicated that pat mutations do not cause accumulation of phenolic compounds in the first 3 weeks of growth, which is before the first lesions appear on the leaves. Since older plants were not tested, it is possible that accumulation of phenolic compounds occurs after 3-weeks of age.

The root is the organ that takes up water and nutrients from the soil. Lateral roots form on primary roots and facilitate water and nutrient uptake from the soil, so they are important for plant growth. It is possible that the pat14 mutation affects roots since pat14 mutants have smaller plant size than wildtypes, so root length and lateral root measurements were performed. Root length assays were performed three times, but data from these assays were significantly different. There were no differences between genotypes at most
time points. The experiments were only for 11 days so looking at root systems at later development stages would be useful. Lateral root assays were done two times and wildtype plants had significantly more lateral roots than pat14 mutants at 9 and 11 days. These results indicate that the pat14 mutation affected lateral root formation but not growth of primary roots, at least at these earlier stages of growth.

The pat14 mutation did not affect the ability of seeds to germinate but did have an effect on germination time. There was a slight but significant difference in the number of seeds germinated after 24 hours, with wildtype having more germinated seeds than either mutant and pat14-1 mutants having more germinated seeds than pat14-2. I conclude that the pat14 mutation caused the seeds to germinate more slowly than wildtype seeds.

Based on seed yield, seed size and the number of seeds per silique, both pat mutants had a stronger phenotype than wildtype plants and pat14-1 plants had a stronger phenotype than pat14-2. pat14 mutant plants were smaller than wildtype, so it is not surprising that pat mutants had fewer siliques than wildtype plants. Fewer siliques would obviously lead to less seed yield. To compound the effect, pat mutants also had fewer seeds per silique which also contributed to reduced seed production in mutants.

In conclusion, a number of phenotypes were detected for plants with homozygous pat14-1 and pat14-2 alleles, indicating that palmitoylation of PAT14 substrates are important in Arabidopsis thaliana growth. My hypothesis was that
*pat14-1* and *pat14-2* mutants have similar phenotypes to each other but are different from wildtype plants. I have demonstrated that, for many of the assays that I conducted, mutants had different phenotypes than wildtype plants. *pat14* mutants have similar phenotypes, but they may differ in intensity of the phenotype even though the mutations were close each other in the *PAT14* gene.

**Replication of Experiments**

Quantitative assays should be repeated at least twice and the results should not be significantly different. If this is the case, the results of the assays can be combined and presented together. In this research, quantitative assays were performed at least two times with the exception of seed size measurements. Since the results were very reproducible, data from silique number, seeds per silique, seed yield and germination time assays were combined and the results of these experiments were presented together.

Characterization of leaf phenotypes was attempted 13 times in 2 years, but were completed in their entirety only two times due to a number of confounding problems including growth chamber malfunctions, problems with plant culture, and use of different growth chambers which did not yield reproducible results. Since the data from these assays were different, they could not be combined and the data from each experimental replication was presented separately.

Assays of root length were repeated three times. Even though the trends between genotypes were similar, these assays were significantly different. The
reason for the difference between assays is unknown since the same growth room was used for all three assays.

**Future Work**

Characterization of the potential functions of the PAT14 protein would be helpful for understanding of roles of palmitoylation in plants. For this reason, more comparisons should be done especially at the molecular level to complement the characterization of the visual phenotypes performed in this research. Determining the subcellular localization of PAT14 protein will be also useful to understand the functions of this protein.

The data resulting from quantitation of leaf phenotypes of Arabidopsis *pat* mutants were not always reproducible. Also, there was no correlation between the results of the two pigment estimation assays. Leaf measurements and pigment assays need to be repeated. Although there are many defense system responses in plants, the accumulation of phenolic compounds was the only one investigated in this research. More plant defense responses, like accumulation of callose, expression of pathogenesis-related genes, and observation of dead cells, should be checked in the *pat* mutant plants.
LITERATURE CITED


yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in *Arabidopsis thaliana*. *Plant Cell Physiol.*, 47, 74-83.


Feng, Y., Davis, N. G. (2000). Akr1p and the type I casein kinases act prior to


The Arabidopsis Information Resource. Genome Assembly. Retrieved, April 05,


Figure 63. Average number of siliques per plant 2 weeks after flowering. The three genotypes were compared using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p\leq0.05$. A) Assay 1; B) Assay 2.
Figure 64. Average number of siliques per plant on 7-week-old plants. The three genotypes were compared using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p\leq0.05$. A) Assay 1; B) Assay 2.
Figure 65. Average number of seeds per silique 3 weeks after flowering. The three genotypes were compared using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p≤0.05. A) Assay 1; B) Assay 2.
Figure 66. Yield of seeds rom wildtype, *pat14-1* and *pat14-2* plants. The three genotypes were compared using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. A) Assay 1; B) Assay 2.
Figure 67. Number of the germinated seeds per genotype. The three genotypes were compared at each time point using Tukey’s range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at p≤0.05. A) Assay 1; B) Assay 2.
Figure 68. Total number of the germinated seeds per genotype. The three genotypes were compared at each time point using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. A) Assay 1; B) Assay 2.
Figure 69. Comparison of lateral roots by genotype. The three genotypes were compared at each time point using Tukey's range test in conjunction with an ANOVA. Different letters indicate that the averages are statistically significant at $p \leq 0.05$. A) Assay 1; B) Assay 2.